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Journal of Invertebrate Pathology 81 (2002) 86–93
Journal of INVERTEBRATE PATHOLOGY
www.academicpress.com

The potential for enhanced fungicide resistance in *Beauveria bassiana* through strain discovery and artificial selection☆

David I. Shapiro-Ilan,* Charles C. Reilly, Michael W. Hotchkiss, and Bruce W. Wood

USDA-ARS, SE Fruit and Tree Nut Research Lab, 21 Dunbar Rd, Byron, GA 31008, USA

Received 28 May 2002; accepted 9 September 2002

Abstract

Our objectives were to determine the (1) natural variation in fungicide resistance among *Beauveria bassiana* strains, (2) potential to increase fungicide resistance in *B. bassiana* through artificial selection, and (3) stability of virulence in selected *B. bassiana* strains. Fungicides included dodine, fenbuconazole, and triphenyltin hydroxide, which are commonly used in pecan and other horticultural crops. Comparison of seven *B. bassiana* strains indicated some are substantially more resistant to fungicides than others; a commercial strain (GHA) was less resistant than all wild strains isolated from pecan orchards. Artificial selection resulted in enhanced fungicide resistance in the GHA strain but not in a mixed wild strain. Removal of selection pressure for three passages did not reduce the enhanced fungicide resistance. Sub-culturing with exposure to fungicides did not affect the GHA strain's virulence to pecan weevil, *Curculio caryae*, larvae, whereas fungicide exposure increased virulence in a mixed wild population of *B. bassiana*.

Keywords: *Beauveria bassiana*; Biological control; *Curculio caryae*; Fungicide; Genetic improvement; Pecan weevil; Resistance; Virulence

1. Introduction

Beauveria bassiana (Balsamo) Vuillemin is a biological control agent that can suppress a variety of economically important pests (Booth et al., 2000; Tanada and Kaya, 1993). Although many agrochemicals are compatible with *B. bassiana* (Goettel et al., 2000; Rosin et al., 1996), survival, growth, or efficacy of *B. bassiana* can be reduced by certain chemical insecticides (Clark et al., 1982; James and Elzen, 2001; Olmert and Kenneth, 1974), herbicides (Gardner and Storey, 1985), and particularly fungicides (Clark et al., 1982; Jaros-Su et al., 1999; Loria et al., 1983; Majchrowicz and Poprawski, 1993; Olmert and Kenneth, 1974; Tedders, 1981; Todorova et al., 1998). Fungicides affect *B. bassiana* differently depending on the particular chemical (Jaros-Su et al., 1999; Olmert and Kenneth, 1974) and timing of application (Jaros-Su et al., 1999). Although effects are more pronounced under laboratory conditions, inhibition of *B. bassiana* by fungicides has also been observed in the field (Clark et al., 1982).

The pecan weevil, *Curculio caryae* (Horn), is a key pest of pecan throughout the Southeastern US (Mizell, 1985). Control recommendations for *C. caryae* currently consist solely of foliar applications of chemical insecticides (e.g., carbaryl), to kill adults (Harris, 1999; Hudson et al., 2002), thus warranting investigation of alternative measures. Several studies have indicated potential for *B. bassiana* to control *C. caryae* (Gottwald and Tedders, 1983; Tedders et al., 1973). One promising approach is to apply *B. bassiana* to suppress *C. caryae* adults as they emerge from soil (Gottwald and Tedders, 1983; Harrison et al., 1993; Shapiro-Ilan et al., unpublished data). However, adult *C. caryae* emergence and control can coincide with late summer fungicide sprays (Hudson et al., 2002). Thus, discovery or development of *B. bassiana* strains that are resistant to fungicides could be beneficial to management programs for *C. caryae* and other insect pests.

Research directed toward increasing fungicide resistance in Hyphomycetes fungi, such as *B. bassiana* and *Metarhizium anisopliae* (Metschnikoff) Sorokin, has consisted of molecular genetic approaches, particularly

☆ Mention of a commercial product does not imply endorsement by the US Department of Agriculture.

* Corresponding author. Fax: 1-478-956-2929.
E-mail address: dshapiro@saa.ars.usda.gov (D.I. Shapiro-Ilan).

0022-2011/02/$ - see front matter © 2002 Elsevier Science (USA). All rights reserved.
PII: S0022-2011(02)00151-9

transformation to benomyl resistance (Bernier et al., 1989; Goettel et al., 1990). Discovery of strains naturally possessing a desired trait and artificial selection are simpler approaches to improving biocontrol potential (Gaugler, 1987; Hoy, 1986) and might be used to overcome fungicide effects on beneficial fungi. *B. bassiana* strains have frequently been screened for virulence (e.g., Harrison et al., 1993; Inglis et al., 1996), yet screening for fungicide resistance has not been investigated. Artificial selection has been used to develop pesticide resistance in other biocontrol agents (Glazer et al., 1997; Hoy, 1986), but the approach has not been applied to *B. bassiana*.

Our objectives were to determine natural variation in fungicide resistance among seven *B. bassiana* strains (six recently isolated wild strains and one commercial strain) and to determine the potential for increasing fungicide resistance in *B. bassiana* through artificial selection. Continuous in vitro sub-culturing of entomopathogens (such as *B. bassiana*) can lead to a reduction in virulence (Tanada and Kaya, 1993). Therefore, our third objective assessed the stability of virulence in *B. bassiana* strains that were selected for fungicide resistance. Our study focused on three primary fungicides used for managing fungal diseases of pecan and the target pest for our virulence tests was *C. caryae*.

2. Materials and methods

2.1. Fungi and fungicides

A list of *B. bassiana* strains and their origins is provided in Table 1. Isolates were obtained from pecan orchards using procedures described by Goettel and Inglis (1997) and determined to be different strains based on their relative virulence to *C. caryae* larvae (Shapiro-Ilan et al., 2002). Stock cultures were stored at −80 °C according to Humber (1997). Sub-culturing of all fungal isolates was accomplished on Sabouraud dextrose agar with 0.2% yeast extract (SDAY) according to procedures described by Goettel and Inglis (1997). In all sub-culturing and experimentation, 0.05% Tween 80 (Fisher Scientific, Fair Lawn, NJ) was used as a surfactant in *B. bassiana* conidia suspensions (Krueger et al., 1992). The three fungicides used in this study were dodine (Dodine 65W, 65% active ingredient, wettable powder, Platte Chemical, Greeley, CO), fenbuconazole (Enable 2F, 240 g/L active ingredient, flowable, Rohm and Haas, Philadelphia, PA), and triphenyltin hydroxide (Supertin, 80% active ingredient, wettable powder, Griffin L.L.C., Valdosta, GA).

Table 1
Origin of *Beauveria bassiana* strains used in this study

Strain	Origin
CX2	Pecan orchard soil, West Helena, AR
G4	Pecan orchard soil, Byron, GA
GHA	Emerald BioAgriculture, Salt Lake City, UT (Mycotrol)
H1	Pecan orchard soil, Clarksdale, MS
H5	Pecan orchard soil, Clarksdale, MS
J3	Pecan orchard soil, Rena Lara, MS
Mix	Pecan weevil adults (4), Byron, GA
VS4	Pecan orchard soil, Barnesville, GA

2.2. Natural variation in fungicide resistance

Fungicide resistance was determined in seven *B. bassiana* strains (CX2, G4, GHA, H1, H5, J3, and VS4, see Table 1) using procedures adapted from Olmert and Kenneth (1974) and Tedders (1981), i.e., measuring zones of inhibition on SDAY plates. All strains were sub-cultured less than three times prior to experimentation. Approximately 1×10^3 conidia in 0.1 ml were spread onto each 90 mm SDAY plate. After one hour, a 2 cm plug was removed from the center of each plate and the well was filled with a solution containing one of the three fungicides. Additionally, a 4.25 cm filter paper disc (Whatman No. 1) was dipped into a beaker containing fungicide solution, allowed to drip until excess moisture was expelled, and placed in the center of the plate (over the well created by the missing plug). In preliminary tests we found that zones of inhibition were more pronounced when a well and filter disc was used relative to when each was used alone (unpublished data). Fungicide solutions used in this experiment were equivalent to the recommended field rate assuming the commonly used application volume of 935 L per hectare (Clark et al., 1982). Specifically, the solutions were 1559.0, 149.9, and 449.8 μg/ml active ingredient of dodine, fenbuconazole, and triphenyltin hydroxide, respectively. The inoculated plates were wrapped in laboratory film (Parafilm "M" American National Can., Menasha, WI) and incubated in darkness at 25 °C. After five days, fungicide effects were determined by averaging the zones of inhibition (in cm) at four points on each plate, i.e., at 0, 90, 180, and 270° (where 0° was a randomly chosen point on one end of the plate). The experiment contained five replicates (plates) of each treatment and the entire experiment was repeated once.

2.3. Artificial selection for fungicide resistance

Artificial selection for fungicide resistance was applied to two strains of *B. bassiana* (GHA and Mix, see Table 1). The Mix strain originated from a combination of conidia derived from four infected *C. caryae* adults. Initially, to determine the levels of selection pressure to apply, we tested the ability of each *B. bassiana* strain to grow at varying levels of fungicide exposure. The fungicides were incorporated into SDAY after the agar solution was autoclaved and had cooled to approximately 50 °C. Concentrations of formulated fungicide

products (per ml agar solution) were 0, 1, 10, 100, 200, and 500 μg/ml for dodine, 0, 0.05, 1, 5, 10, and 20 μg/ml for fenbuconazole, and 0, 0.05, 0.5, 1, 5, and 10 μg/ml for triphenyltin hydroxide; these concentrations were chosen based on results of preliminary testing. Suspensions containing 1×10^3 conidia in 0.1 ml were spread onto each 90 mm SDAY plate. After 48 h of incubation at 25 °C, fungal survival was determined under a dissecting microscope (ca. 30×) on each plate by averaging the number of colony forming units (CFUs) (Goettel et al., 2000) in three randomly selected 1 cm^2 areas. There were five replicates (plates) for each fungicide-strain combination and the entire experiment was repeated once. In addition to providing a baseline for choosing selection regimes, this experiment facilitated comparison among the fungicides for their relative abilities to inhibit fungal strains.

Selection regimes were initiated through repeated sub-culturing of *B. bassiana* strains on SDAY mixed with fungicides. The fungicide concentrations that were initially chosen for exposure to *B. bassiana* were based on dose–response tests described above (Fig. 1). The goal was to choose the highest concentration that would support at least some *B. bassiana* growth. For the Mix strain these concentrations (of fungicide per ml agar solution) were 200, 20, and 5 μg/ml of formulated dodine, fenbuconazole, and triphenyltin hydroxide, respectively. For the GHA strain the concentrations were 10, 1, and 1 μg/ml of formulated dodine, fenbuconazole, and triphenyltin hydroxide, respectively. For each strain and fungicide, one colony from each of three fungicide-agar plates was randomly chosen for sub-culturing in the subsequent round of selection. After seven passages, the fungi exposed to fungicides were divided; one group was sub-cultured with selection pressure (fungicide exposure) for an additional three passages (= the "selected" population). The other "relaxed-selection population" was sub-cultured for three passages without selection pressure (on un-amended SDAY). The relaxed-selection population was created to indicate whether or not fungicide resistance (if it had developed) would remain stable when selection pressure was removed. The selected and relaxed-selection populations, as well as a non-selected (control), were tested for fungicide resistance by measuring zones of inhibition as described previously. The non-selected control was stored (at −80 °C) during sub-culturing of the selected and relaxed-selection populations, but was sub-cultured one time on SDAY in parallel with the last (10th) passage of the other populations so that age of the culture was excluded as a factor.

2.4. Stability of virulence in selected populations

A soil cup assay, based on procedures described by Harrison et al. (1993) and Shapiro-Ilan (2001), was used

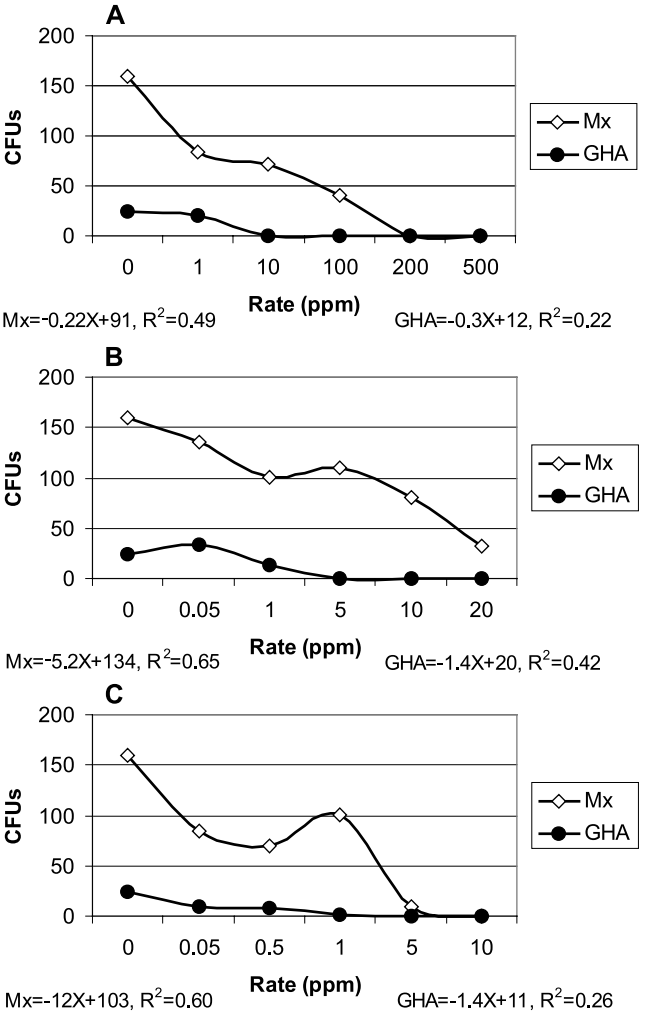

Fig. 1. Mean number of *Beauveria bassiana* colony forming units (CFUs) on agar plates mixed with varying rates of fungicides (A) dodine, (B) fenbuconazole, and (C) triphenyltin hydroxide. Mx, mixed wild *B. bassiana* strain; GHA, *B. bassiana* GHA strain.

to compare virulence of selected and non-selected populations of each *B. bassiana* strain (Mix and GHA). Cups (3–4 cm i.d., 3.5 cm deep) contained 10 g oven-dried soil from the USDA-ARS pecan orchard (Byron, GA) and contained one larva each. The soil was a loamy sand with the percentage sand:silt:clay = 84:10:6, pH = 6.1, and organic matter = 2.8% by weight. Pecan weevil larvae (4th instar), collected from infested nuts on the USDA-ARS Research Station (Byron, GA), were stored in sterile (autoclaved) soil at 25 °C for two weeks, at which time diseased larvae were removed. Remaining larvae were then stored at 4 –10 °C (Shapiro-Ilan, 2001) until use.

Fungi were pipetted in 1.4 ml onto the soil surface of each cup so the final moisture was standardized at field capacity (14%). The application rate was approximately 1.59×10^4 conidia per cm^2 (6.36×10^3 conidia per gram of soil). After inoculation, cups were incubated at 25 °C,

and mortality and signs of mycosis (Tanada and Kaya, 1993) were recorded every one to three days beginning 5 days post-inoculation. Preliminary experimentation indicated observations prior to 5 days were not necessary. The rates of application were based on previous laboratory experiments that showed differences among strains at similar rates (Shapiro-Ilan et al., 2002). A non-treated (water only) check was included. Age of the fungal culture was removed as a factor as described previously. The experiment contained three replicates of 10 cups per treatment and the entire experiment was repeated once.

2.5. Data analysis

Natural variation in fungicide resistance was analyzed by comparing average zones of inhibition among treatments (within each fungicide) using analysis of variance (ANOVA) and Tukey's multiple range test (SAS, 1996). Likewise, for artificial selection experiments, zones of inhibition (within each fungicide and *B. bassiana* strain) were compared using ANOVA and Tukey's test; differences between the two strains (GHA and Mix) were compared with *t* tests (SAS, 1996). The relationship between fungicide concentration and *B. bassiana* CFUs was subjected to linear regression analysis. Confidence intervals (95%) were calculated for the slopes (CFUs/fungicide concentration) and used to compare relative inhibition caused by the different fungicides; lack of overlap indicates significant differences. In the virulence assays, mean percentage mortality and mycosis were arcsine transformed (Southwood, 1978) and evaluated with repeated measures analysis (Proc Mixed), and (if the *F* value was significant), further differentiation was obtained through LSMEANS (SAS, 1996). The α-level for all analyses was 0.05.

3. Results

3.1. Natural variation in fungicide resistance

Natural levels of fungicide resistance to all fungicides varied significantly among *B. bassiana* strains (Fig. 2) ($F = 346.3$, 1246.8, and 117.1 for dodine, fenbuconazole, and triphenyltin hydroxide tests, respectively, df = 6, 69, $P = 0.0001$ for all fungicide tests). For all fungicides, the GHA strain had lowest resistance among strains and the VS4, G4, and J3 strains were consistently in the most resistant grouping (based on zones of inhibition) (Fig. 2). The H1 strain was less resistant to fenbuconazole than four other strains, and the H1 and H5 strains were less resistant than four other strains to triphenyltin hydroxide (Fig. 2).

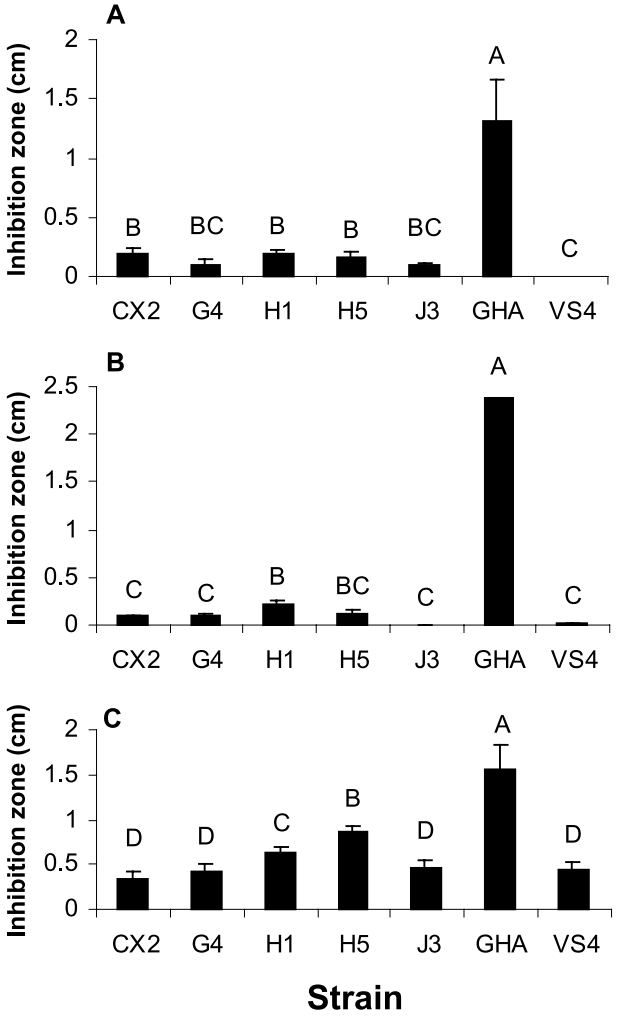

Fig. 2. Resistance of *Beauveria bassiana* strains to fungicides (A) dodine, (B) fenbuconazole, and (C) triphenyltin hydroxide, as measured by mean (+SE) zones of inhibition. Different letters above bars indicate statistical significance (Tukey's test $\alpha = 0.05$). See text for origin of isolates.

3.2. Artificial selection for fungicide resistance

In all fungicide-*B. bassiana* combinations, a significant ($P < 0.05$) negative linear relationship was observed between fungicide concentration and fungal survival (as measured by CFUs on SDAY) (Fig. 1). Confidence intervals around slopes in the Mix strain were −0.137 to −0.321, −3.68 to −6.67, and −8.62 to −16.34 for dodine, fenbuconazole, and triphenyltin hydroxide tests, respectively, indicating inhibition was greatest from triphenyltin hydroxide followed by fenbuconazole (dodine was least inhibitory). Confidence intervals in the GHA strain were −0.009 to −0.055, −0.768 to −1.97, and −0.582 to −2.32 for dodine, fenbuconazole, and triphenyltin hydroxide tests, respectively, indicating inhibition was greater from fenbuconazole or triphenyltin hydroxide than from dodine.

In the GHA strain, selected and relaxed-selection populations exhibited greater resistance to all fungicides compared with the non-selected population (Fig. 3) ($F = 135.8$, df $= 2, 24$, $P = 0.0001$; $F = 15.5$, df $= 2, 20$, $P = 0.0001$; $F = 102.3$, df $= 2, 24$, $P = 0.0001$, for dodine, fenbuconazole, and triphenyltin hydroxide, respectively). In the Mix strain, no differences were detected among selected, relaxed-selection, and non-selected populations except that the relaxed-selection population was less resistant to fenbuconazole than the selected population (Fig. 3) ($F = 2.1$, df $= 2, 24$, $P = 0.14$; $F = 4.0$, df $= 2, 22$, $P = 0.03$; $F = 2.0$, df $= 2, 24$, $P = 0.16$, for dodine, fenbuconazole, and triphenyltin hydroxide, respectively).

Fungicide resistance was greater in the Mix strain relative to the GHA strain in 6 out of 9 comparisons (Fig. 3) ($P < 0.05$). In all non-selected populations fungicide resistance was greater in the Mix strain than the GHA strain, but no difference was detected between strains after they had been selected 10 passages for dodine or triphenyltin hydroxide resistance (Fig. 3). Contrarily, fenbuconazole resistance remained higher in the Mix strain than the GHA strain even after artificial selection.

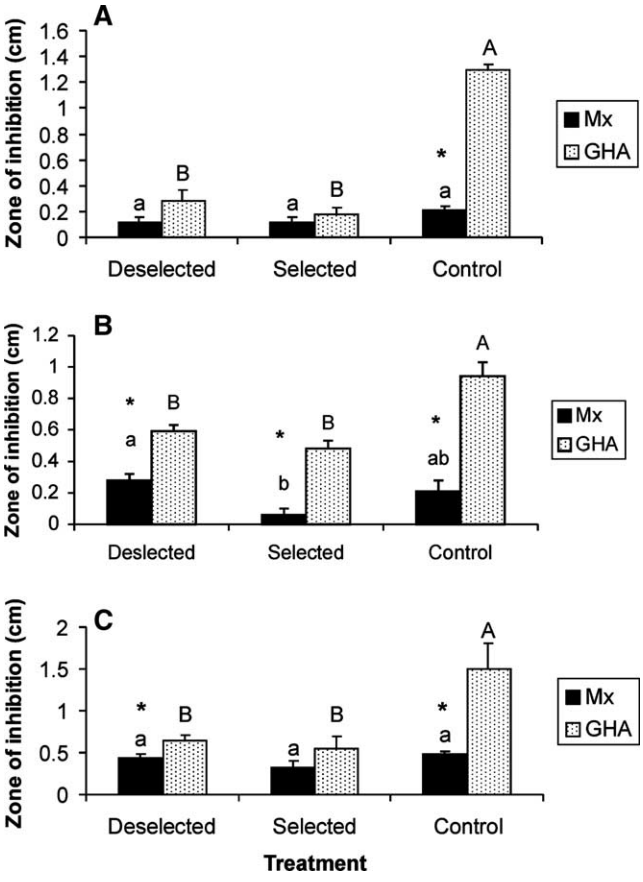

Fig. 3. Resistance of selected, relaxed-selection, and non-selected (control) *Beauveria bassiana* populations to fungicides (A) dodine, (B) fenbuconazole, and (C) triphenyltin hydroxide, as measured by mean (+SE) zones of inhibition. Mx, mixed wild *B. bassiana* strain; GHA, *B. bassiana* GHA strain (Mycotrol). Different letters above bars indicate statistical significance within strain (Tukey's test $\alpha = 0.05$). An asterisk indicates statistical difference between strains (within treatments).

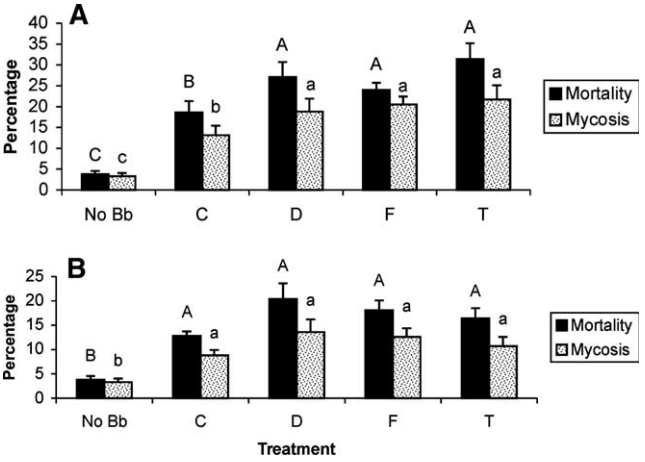

Fig. 4. Mean (+SE) percentage mortality and mycosis in *Curculio caryae* larvae averaged 14 days following exposure to (A) mixed wild *Beauveria bassiana* strain, (B) *B. bassiana* GHA strain (each at 6.36×10^3 conidia per gram of soil). Fungicide selection regimes: No Bb, water only; C, non-selected control; D, dodine selected; F, fenbuconazole selected; T, triphenyltin hydroxide selected. Different uppercase (for mortality) or lower case (for mycosis) letters above bars indicate statistical significance (LSMEANS, $\alpha = 0.05$).

3.3. Stability of virulence in selected populations

For the Mix strain of *B. bassiana*, all fungal populations selected for fungicide resistance caused higher mortality and mycosis in *C. caryae* larvae than the non-selected population, and all *B. bassiana* treatments caused greater mortality than the non-treated check (Fig. 4A) ($F = 31.2$, df $= 4, 176$ $P = 0.001$; $F = 20.7$, df $= 4, 176$, $P = 0.0001$, for mortality and mycosis, respectively). For the GHA strain, no differences were detected among *B. bassiana* populations though all of them caused greater mortality and mycosis than the check (Fig. 4B) ($F = 13.9$, df $= 4, 176$ $P = 0.001$; $F = 6.7$, df $= 4, 176$, $P = 0.0001$, for mortality and mycosis, respectively).

4. Discussion

We observed substantial natural variation in fungicide resistance among *B. bassiana* strains. Thus, strain discovery has the potential to overcome inhibitory effects of concurrent *B. bassiana* and fungicide applications. For example, for pecan pest management, we demonstrated that *B. bassiana* strains such as VS1, G4, and J3 are likely to be less affected by fungicide use than other strains, e.g., GHA. Contrary to our results Olmert

and Kenneth (1974) observed only minimal variation in resistance to 6 fungicides among 5 *B. bassiana* isolates (determined by zone of inhibition assays), although they reported great variation in fungicide resistance among isolates of *Verticillium* spp. Perhaps, if they had tested other isolates or fungicides they would have observed results similar to ours. The commercially available GHA strain was less resistant than the other strains we isolated from pecan orchards. It is conceivable that the greater resistance observed in the wild isolates was due to previous exposure (and thus selection) in the field. Indeed, Roush (1990) advocated that if one wants to obtain pesticide resistant biocontrol agents one should search areas of heavy pesticide use.

A number of studies indicate that fungicide effects on *B. bassiana* vary depending on the chemical used (Clark et al., 1982; Jaros-Su et al., 1999; Majchrowicz and Poprawski, 1993; Olmert and Kenneth, 1974; Tedders, 1981). For example, research indicates inhibition from benomyl tends to be relatively harsh (Olmert and Kenneth, 1974; Tedders, 1981); whereas certain copper based fungicides have a relatively mild effect (Jaros-Su et al., 1999; Majchrowicz and Poprawski, 1993; Olmert and Kenneth, 1974). Similar to our results, Tedders (1981) reported the detrimental effects of triphenyltin hydroxide on *B. bassiana* to be substantially greater than the effects of dodine. The tolerance of *B. bassiana* to dodine facilitates the chemical's use in selective media (Chase et al., 1986).

Our results indicate that fungicide resistance in *B. bassiana* can be obtained through artificial selection. Artificial selection enhanced fungicide resistance in the GHA strain but we did not detect any change in the Mix strain. One may have expected an opposite trend because the Mix strain was a combination of several isolates and therefore likely possessed greater genetic variability than the GHA strain; the potential for artificial selection generally increases with genetic variation (Gaugler, 1987; Roush, 1990). However, changes in laboratory cultures may also occur due to mutation (Tanada and Fuxa, 1987), which may have played a major role in these experiments. Additionally, the non-selected Mix strain was far more resistant than the GHA strain to begin with and thus changes in resistance (if they did occur) were inherently more subtle and difficult to detect.

Enhanced or superior beneficial traits in biological control agents can be lost or reduced when selection pressure is removed (Hoy, 1986; Shapiro et al., 1996). The increased fungicide resistance detected in the GHA strain remained stable without selection pressure for at least three passages. Although three passages is sufficient for many culture purposes, further research is required to determine if the fungicide resistance will remain stable for a longer period. The lower resistance to fenbuconazole observed in the relaxed-selection Mix population compared with the selected population is suggestive of some deterioration, yet the relaxed-selection population's resistance was not different from the control.

Several studies indicate that the level of fungicide inhibition of *B. bassiana* observed under laboratory conditions may be substantially reduced under field conditions (Clark et al., 1982; Jaros-Su et al., 1999; Loria et al., 1983; Mietiewski et al., 1997). Nonetheless, fungicide inhibition of *B. bassiana* has been observed under field conditions. For example, Clark et al. (1982) reported reduced field suppression of the Colorado potato beetle adults, *Leptinotarsa decemlineata* (Say), by *B. bassiana* when applications were made in conjunction with mancozeb, Jaros-Su et al. (1999) observed reduced *B. bassiana* survival under field conditions due to the presence of mancozeb or chlorothalonil, and Mietkiewski et al. (1997) reported reduced *B. bassiana* activity in soil receiving benomyl relative to plots without fungicide application.

There is potential for fungicide sprays to inhibit *B. bassiana* suppression of *C. caryae* under field conditions. One could argue that the level of contact between *B. bassiana* and fungicides would be minimal because the fungicides are applied to the tree canopy whereas *B. bassiana* control of *C. caryae* is most likely to succeed as a soil application (Shapiro-Ilan, unpublished). However, research indicates that rainfall can rapidly wash fungicides from the tree (Reynolds et al., 1994), breakdown in soil can be slow (Kannan and Lee, 1996), and at least some *B. bassiana* can be found above the soil surface (Doberski and Tribe, 1980). Contact between the two agents may also be reduced due to timing of applications. Recommendations for scab, *Cladosporium caryigenum* (Ellis and Langl.) Gottwald, control (the most important pecan disease) generally focus on fungicide sprays during pre-shell hardening periods (e.g., June and July), usually before weevils emerge (Brenneman et al., 1999). However, fungicide spraying may also run into August (Hudson et al., 2002), which can overlap with peak *C. caryae* emergence (Harris, 1976; 1999). Furthermore, the persistence and toxicity of fungicide breakdown products to *B. bassiana* has yet to be determined. Field trials will be required to ascertain whether fungicide sprays can indeed interfere with *B. bassiana* applications. In any case, if it is determined that fungicide resistance is needed in *B. bassiana* for pecan management we have demonstrated that it can be obtained through strain discovery or artificial selection.

A number of studies have reported virulence reductions following in vitro sub-culture in various entomopathogenic fungi (Aizawa, 1971; Morrow et al., 1989; Schaerffenberg, 1964). Similarly, selection for one beneficial trait in a biocontrol agent can lead to reduction in other beneficial traits (Hoy, 1986). However, other studies have shown virulence to remain stable after numerous passages, e.g., Brownbridge et al. (2001)

observed no virulence changes in *B. bassiana* GHA strain after 15 passages in vitro. Similarly, we observed no change in virulence in the GHA strain following repeated in vitro sub-culturing (10 passages) on medium with fungicides. Moreover, we observed increased virulence in the Mix strain of *B. bassiana*.

Synergistic effects on insect suppression have been observed when *B. bassiana* was applied with certain pesticides. For example, Quintela and McCoy (1998) demonstrated synergy between imidacloprid and *B. bassiana* for control of the diaprepes root weevil, *Diaprepes abbreviatus* (L.), and Todorova et al. (1998) reported synergized insecticidal activity vs *L. decemlineata* when *B. bassiana* was combined with diquat. The increased virulence we observed in the Mix strain, however, is markedly different from observations of synergy. Studies demonstrating synergy involve a dual effect of the fungus and pesticide on the insect when the control agents are applied simultaneously. Contrarily, we observed a direct effect on *B. bassiana* resulted in enhanced insect suppression when the fungus was applied alone (without pesticides). For reasons that are unclear, factors associated with fungicide exposure resulted in increased *B. bassiana* virulence. The mechanism of increased virulence and the extent it may be applied to other fungi, pesticides, and insects merit investigation.

Acknowledgments

We thank Kathy Halat, Wanda Evans, and Ronald Epps Jr. for technical assistance, Drs. Rosalind James and Stephan Jaronski for helpful comments on assay procedures and reviewing an early draft of the manuscript, and Emerald BioAgriculture for providing *B. bassiana* GHA strain.

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